THE PHYSIOLOGY OF INSECT DIAPAUSE. IX. THE CYTOCHROME OXIDASE SYSTEM IN RELATION TO THE DIAPAUSE AND DEVELOPMENT OF THE CECROPIA SILKWORM [1]

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During the pupal diapause the respiratory metabolism of the Cecropia silkworm proceeds at a low and relatively constant rate which, except in the case of the intersegmental muscles of the abdomen, is insensitive to carbon monoxide and cyanide. However, with the termination of diapause and the initiation of adult development, a carbon monoxide- and cyanide-sensitive respiration appears and increases progressively, being superimposed on the carbon monoxide-stable respiration of diapause. It was concluded from these and other observations that the metabolism of the developing insect is largely mediated by the cytochrome oxidase system while that of the diapausing pupa is not (Schneiderman and Williams, 1954).

But respiratory measurements in themselves can provide only circumstantial evidence that the coupling of metabolism to cytochrome function is causally related to the termination of diapause and the development which follows. The problem is basically morphogenetic in character and therefore demands solution in morphological terms. Is the change in terminal oxidase coincidental, or is there an obligatory coupling between the function of the cytochrome oxidase system and the actual development of the insect? The present study was designed to answer this question by direct observations of the effects of carbon monoxide on the growth of the Cecropia silkworm during successive stages of metamorphosis.

Materials and Methods

1. Experimental animals

Experiments were performed on embryos, mature larvae, pupae, developing adults, and adults of the giant silkworm *Platysamia cecropia*. The pupae were of three types: (a) Normal diapausing pupae removed from their cocoons and stored continuously at 25° C. ("unchilled diapausing pupae"). (b) Diapausing pupae such as the preceding, except that the brains had been removed and plastic windows established in the facial region and at the tip of the abdomen ("brainless diapausing pupae"). (c) "Previously chilled diapausing pupae"—animals that had been stored at 5° C. for approximately six months and provided with plastic terminal abdominal windows. As previously reported (Williams, 1946), prolonged exposure to low

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temperature renders the brain competent to secrete its hormone and results in the initiation of adult development approximately two weeks after such pupae are returned to 25° C.

In previously chilled pupae provided with plastic windows, the heart-beat and the initiation and day-to-day progress of adult development could be observed directly under the dissecting microscope. As has been emphasized in the previous papers of this series, the visible initiation of adult development is an event of special significance since it signals the end of the months of pupal diapause. Table I records the time sequence of adult development as observed beneath facial and terminal abdominal windows at 25° C., from the first visible signs of hypodermal retraction to the emergence of the adult moth approximately 22 days later. The table records the average tempo of development of a large series and permits one to estimate the stage of development to within ± 12 hours in the vast majority of individuals.

TABLE I

Time-table for the development of male chilled Cecropia at 25° C. as witnessed in pupae equipped with facial and abdominal windows *

Day	*Characters*
0	Initiation of hypodermal retraction just ventral to imaginal disc of genitalia; no retraction elsewhere.
1	Hypodermal retraction under terminal window extends half way up each side; the aedeagus and harpal lobes have tripled in size and migrated slightly toward center of window; hypodermal retraction under facial window has occurred only along posterior margin and is restricted to the midline and the lateral angles; *no retraction of leg hypodermis.*
2	*Initiation of retraction of leg hypodermis,* harpes show considerable enlargement and sharply defined outer edges; beginning of midventral fold between harpes; the aedeagus has migrated about half way to center of window.
3	Facial retraction nearly complete; eye lobes partially visible; terminal retraction complete except dorsally; mid-ventral fold of genitalia extends dorsally to aedeagus; harpes show considerable molding and beginning of subdivision into upper and lower lobes; tips of dorsal harpal lobe slightly forked.
4	Facial and terminal retraction complete; eye lobes well developed but unpigmented; further subdivision of harpes into dorsal and ventral lobes; aedeagus has a cone-shaped, transparent, undivided, membranous tip.
5	Palps and "stalks" of antennae visible for first time. Harpes considerably enlarged and show well developed upper and lower fleshy, semi-transparent lobes; no pubescence; no eye pigment.
6	Membranous tip of aedeagus subdivided into two or three semi-transparent processes; harpal lobes with sharp edges; extremely delicate transparent pubescence along outer edge of upper harpal lobes; no pubescence of lower lobes; no eye pigment.
7	Initiation of pink eye pigment; transparent pubescence now extends along outer edge of lower harpal lobes; genitalia deeply telescoped into preceding segment.
8	Generalized reddish brown eye pigment; genitialia fully formed but fleshy and unpigmented; pubescence generally distributed over outer side of all harpal lobes, but longer and "silky" along edge of upper lobes.
9	Dark reddish brown eye pigment; long silky hairs on upper harpal lobes and shorter silky hairs on lower lobes.
10	Dark brown eye pigment; long silky hairs on all harpal lobes; membranous tip of aedeagus with fleshy spine.
11	No further change.

(Continued on next page)

* The same time-table may also be used for the female insect, save for the characteristics pertaining to the male genitalia. Characters printed in italics are visible without windows and can be seen by moistening the overlying cuticle with 70 per cent alcohol. The adult genitalia of Cecropia have been described and figured by Michener (1952).

TABLE I—*Continued*

Day	*Characters*
12	Tan streak of pigment present on each side of mouth opening; white hairs on upper harpal lobes and on face; earliest tan pigment on genitalia along surface of gnathos and on ridge connecting upper and lower harpal lobes on each side.
13	*Tarsal claws black;* facial cuticle with pale diffuse tan pigmentation; coarse white hairs on harpes; tannish pigmentation of triangular plate (annulus) below base of aedeagus, the pigment extending bilaterally to lower tip of lower harpal lobes; the latter, in turn, show minute black punctate spots; tip of aedeagus dark brown; tan pigmentation of upper harpal lobes; spine on membranous tip of aedeagus still transparent.
14	Spine on tip of aedeagus black; *black, fully-formed antennal barbs.*
15	
16	Persistence of coarse white hairs.
17	*Three black spots along posterior edge of each forewing;* the coarse white hairs on genitalia show initiation of pale pink pigmentation.
18	*Generalized but incomplete wing pigmentation;* red, pink, and white hairs on genitalia; cuticle "soft" only in region of forewings.
19	*Complete wing pigmentation; softening of cuticle extends to dorsum of abdomen.*
20	*Cuticle "soft" throughout but not crisp;* moulting fluid partially absorbed under facial and abdominal windows.
21	*Cuticle crisp throughout; moulting fluid fully resorbed except under abdominal window; cuticle semi-transparent.*
22	*Animal distended; adult emergence.*

2. *Experimental methods*

All experiments were performed at 25° C. Three techniques were utilized in the management of the various gas mixtures:

a. In the *flow method* one or more insects were enclosed in a glass tube through which an approximately streamlined and steady flow of a specific gas mixture was maintained. The mixtures were prepared in pressure cylinders and analyzed prior to use.

b. In the *static pressure method* each animal was placed in a shell vial and the latter loosely plugged with cotton. The vial was then sealed in an individual 2.5-liter air-filled steel chamber and compressed with a specific gas, the pressure being read on a gauge calibrated in pounds per square inch. Alternatively, one or more animals were enclosed in a 3.5-liter air-filled polymethyl methacrylate (Lucite) chamber and compressed with a specific gas. The oxygen tension in the chambers was that of air (20.9 per cent of an atmosphere), while the pressure of the added gas was the gauge pressure. After storage at 25° C. for specific periods the chambers were slowly decompressed, the animals returned to air, and observations continued over a period of several weeks.

c. In the *constant composition pressure method,* a series of insects was placed in a Lucite holder so that their terminal abdominal windows faced uppermost; the holder was then enclosed in a 3.5-liter air-filled Lucite chamber (Fig. 1). The animals were therefore visible through the transparent wall of the chamber and could be studied under the dissecting microscope. A glass trough containing 10 per cent NaOH was placed in the chamber for the purpose of absorbing carbon dioxide. Control experiments revealed that the reaction of carbon monoxide with the concentrated alkali to produce formate occurred so slowly that it did not detectably dimin-

ish the total carbon monoxide pressure. The chamber also contained a calibrated capillary barometer for the measurement of absolute pressure (Schneiderman and Feder, 1954). The air-filled tank was compressed with carbon monoxide, the final pressure being recorded on the tank gauge and the capillary barometer. The latter was read at three-day intervals and the oxygen consumed by the animals replaced by the addition of a corresponding amount of oxygen. On each such occasion a sample of gas was removed and analyzed (Scholander and Roughton, 1943), thus

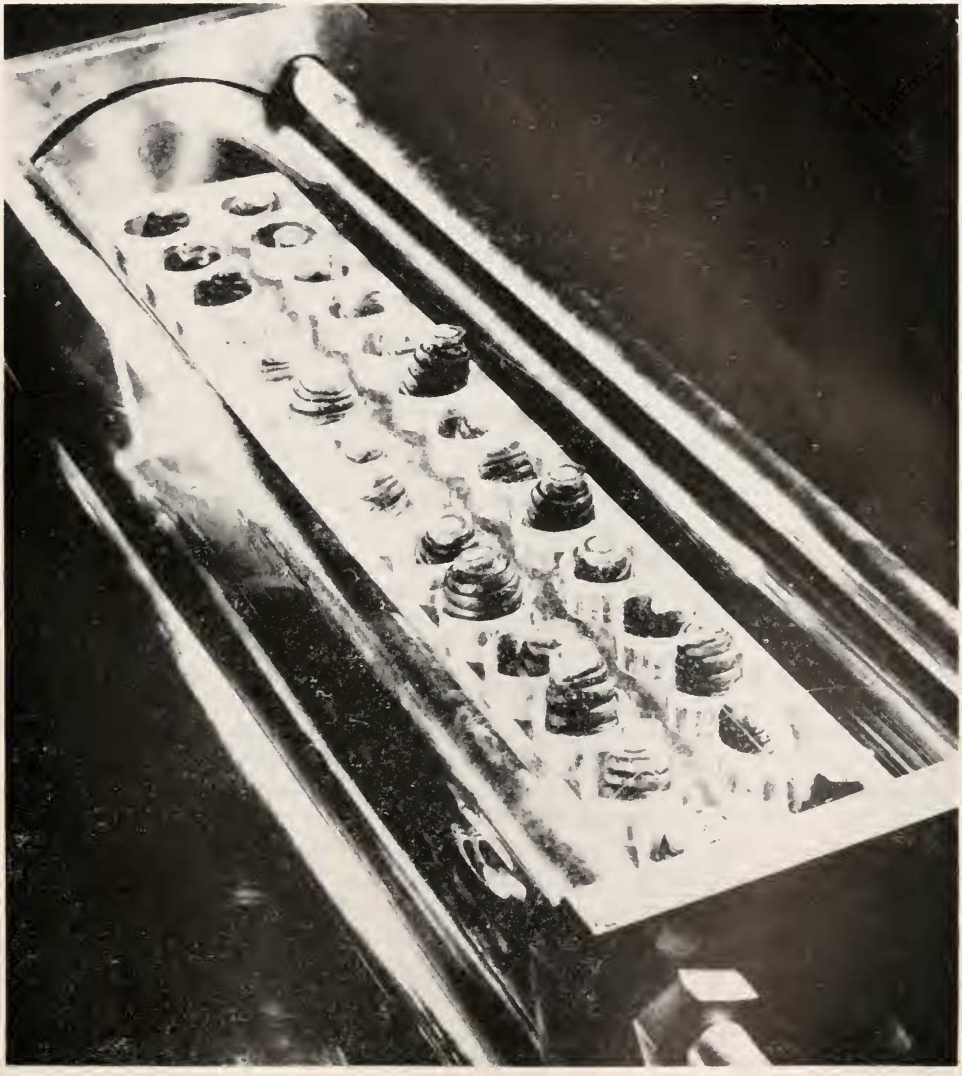

FIGURE 1. Transparent pressure chamber for studying the effects of high pressures of carbon monoxide on the day-to-day progress of development. Thirty animals have been equipped with plastic windows and sealed within the chamber in the presence of five atmospheres of carbon monoxide.

giving double assurance that the oxygen tension in the chamber remained within the desired limits. At the termination of the exposure period the chamber was slowly decompressed, the animals returned to air, and observations continued.

3. Experimental gases

The compressed gases (oxygen, nitrogen, and carbon monoxide) were handled as previously described (Schneiderman and Williams, 1954). In one series of experiments extremely pure carbon monoxide was prepared (*ibid.*). Since the latter was indistinguishable from alkali-washed carbon monoxide in its effects on growth, the less expensive commercially available carbon monoxide was utilized in subsequent experiments.

Results

1. Effects of high pressures of nitrogen

Chilled and unchilled diapausing Cecropia pupae and post-diapausing animals at several stages in adult development were placed in individual air-filled 2.5-liter steel or Lucite chambers and compressed with from 4 to 7 atmospheres of nitrogen. The static pressure technique was utilized and each experiment continued for 21 days. Under this treatment the animals behaved as in air at atmospheric pressure. Spontaneous movements of the abdomen and the beating of the heart continued without interruption. Moreover, the rate of adult development was the same as in air at one atmosphere, and the resulting adults were normal in all respects.

From these control experiments we learn that pressures up to seven atmospheres of an inert gas such as nitrogen are without detectable effects on the adult development of Cecropia. It is also clear that the 525 cc. of oxygen initially present in each air-filled chamber was sufficient to permit a pupa to undergo normal adult development without interference from oxygen lack or from the accumulation of metabolic carbon dioxide during the 21-day period of confinement.

2. Effects of carbon monoxide on diapausing pupae

Diapausing pupae and brainless diapausing pupae were exposed to carbon monoxide by all three of the above-mentioned experimental methods. When the carbon monoxide/oxygen ratio was increased above 10:1, spontaneous movements of the abdomen showed considerable reduction in both amplitude and frequency. Residual extremely feeble movements, occasionally detectable even at 15:1 carbon monoxide/oxygen, completely disappeared after further increase in the ratio. When decompressed and returned to air, normal abdominal motion reappeared within a few hours. In contrast to the paralysis of the intersegmental muscles, the heart continued to beat normally throughout the 21 days of exposure to carbon monoxide even when the carbon monoxide/oxygen ratio was 25:1.

It will be recalled that diapausing pupae initiate adult development after continuous storage at 25° C. for five months or longer (Williams, 1946). This behavior was unimpaired by three weeks of prior exposure to high pressures of carbon monoxide. Evidently, within the diapausing insect the viability of neither the pupal tissues, nor the anlagen of the adult tissues, nor the endocrine organs themselves is dependent on enzymes inhibited by carbon monoxide.

3. Inhibition of wound healing in diapausing pupae by carbon monoxide

Although brainless diapausing pupae are incapable of initiating adult development (Williams, 1946), they retain the ability to repair integumentary wounds. One can study this process to good advantage by removing a disc of hypodermis plus overlying cuticle and covering the wound with a plastic window, the latter being sealed in place with melted paraffin. Spindle-shaped blood cells promptly adhere to the window and begin to string out tenuous cytoplasmic processes. The latter interlace and form a fenestrated tissue which, after 4 or 5 days, is transformed into a transparent, shiny membrane. Meanwhile, the hypodermis begins to close in around the margins of the wound, accompanied by minute tracheae and tracheoles. A continuation of this centripetal growth leads to a central closure of the wound after about 10 to 14 days.

In order to ascertain the effects of carbon monoxide on wound healing, the following experiment was performed. From a series of six previously chilled diapausing pupae the brains were removed and facial and abdominal windows established in each individual. Two days after the operation, three animals were placed in a transparent 3.5-liter air-filled Lucite tank and compressed *via* the static pressure method with five atmospheres of carbon monoxide (carbon monoxide/oxygen ratio of 25:1). The other three animals served as controls and were maintained in air. Each individual was examined daily under the dissecting microscope for signs of regeneration. After a total of 13 days the control group in air had completely repaired the wounds under both facial and abdominal windows. By contrast, the experimental group in carbon monoxide showed no evidence of repair. But when decompressed and returned to air, repair began at once and was completed within 13 days.

Thus, it is clear that even in the diapausing pupa the localized morphogenesis inherent in the repair of a wound is completely inhibited by carbon monoxide.

4. Inhibition of adult development by carbon monoxide

All three of the techniques for the administration of carbon monoxide were utilized in a study of the adult development of previously chilled pupae and of animals that had already initiated adult development. The progress of development in each individual was judged by observations of its genitalia, the day-to-day changes being compared with the normal tempo already defined (Table I). Each experiment was continued for 21 days.

As recorded in Table II it is of special interest that during exposure to carbon monoxide the termination of pupal diapause, as signalled by the onset of adult development, was blocked or greatly delayed. Moreover, individuals which already showed early adult development at the outset of the experiment remained alive in most cases, but further development was either prevented or greatly inhibited.

It is also clear from Table II that the degree of inhibition was a function, not of the carbon monoxide concentration alone, but of the carbon monoxide/oxygen ratio. When the latter was higher than 20:1, development was completely or almost completely blocked. Such animals, when returned to air, promptly resumed normal development where they had left off and produced normal adult moths. However, when development was incompletely blocked in carbon monoxide/oxygen ratios less than 20:1, the insects, upon return to air, continued in a pattern of ab-

TABLE II

Effects of twenty-one days exposure to various carbon monoxide/oxygen ratios on previously chilled Cecropia pupae and on animals at specific stages of adult development

CO/O_2 ratio	Gas content of chamber (atmospheres)	Stage of development at outset	Number of animals	Number of survivors	Average rate of development in CO as % of rate in air	Development after return to air
33:1	6.7 CO +1 air	Previously chilled pupae	6	6	(0.5)	6 normal adults
		<5–25% development	9	8	1	5 normal adults; 3 died
25:1	5 CO +1 air	Previously chilled pupae	8	5	(2)	2 normal adults; 3 died
		<5–25% development	6	5	2	3 normal adults; 2 died
20:1	4 CO +1 air	Previously chilled pupae	8	5	2	No data
19:1	0.95 CO +0.05 O_2	<5–25% development	6	6	11	3 abnormal adults; 1 normal adult; 2 died
		26–50% development	3	3	12	3 slightly abnormal adults
		70% development	1	1	18	1 slightly abnormal adult
15:1	3 CO +1 air	Previously chilled pupae	2	2	(3)	1 abnormal adult; 1 normal adult
		<5–25% development	2	2	15	1 abnormal adult; 1 normal adult
10:1	4 CO +0.2 O_2 +1 air	Previously chilled pupae	8	8	(7)	3 abnormal adults; 3 normal adults; 2 died
		<5–25% development	10	10	14	6 abnormal adults; 4 died
		25–50% development	4	2	25	2 died at 70% stage of development
5:1	1 CO +1 air	Previously chilled pupae	2	2	(15)	No data
1:1	1 CO +0.8 O_2 +1 air	<5–25% development	3	0	70	3 died at 70% stage of development
		Total	78			

Parentheses () indicate that one or more individuals initiated development in the presence of carbon monoxide.

normal development and produced adult moths with various abnormalities. The latter included defective scales, hairs, and pigmentation, along with incomplete or malformed eyes, legs, antennae, and genitalia.

The endocrine competency of the brain itself was found to be unaffected by exposure to carbon monoxide. Thus, brains removed from previously chilled pupae after 21 days of exposure to 25 : 1 carbon monoxide/oxygen retained their activity and evoked adult development when implanted into brainless diapausing pupae.

5. *Effects of carbon monoxide on mature larvae*

Mature larvae at the outset of spinning were exposed to carbon monoxide by the static pressure method for one to six days. In control experiments four atmospheres of nitrogen was substituted for the carbon monoxide.

The results recorded in Table III show that neither the behavior nor the viability of the caterpillars was affected by four atmospheres of nitrogen. By contrast, no individual was able to survive exposure to 33 : 1 carbon monoxide/oxygen for as long as five days. Moreover, in the presence of carbon monoxide/oxygen ratios as low as 1 : 1, the spinning of a normal cocoon was inhibited, the insect either failing to spin or spinning only a flat sheet of silk.

TABLE III

Effects of various carbon monoxide/oxygen ratios on mature fifth instar Cecropia larvae

$CO:O_2$ ratio	Gas content of chamber (atmospheres)	Duration of exposure (days)	Type of spinning behavior in chamber	Type of spinning behavior after removal from chamber	Pupated
—	1 air	2	Normal cocoon	Normal cocoon	+
—	4 N_2+1 air	2	Normal cocoon	Normal cocoon	+
33:1	6.7 CO+1 air	5	None	Dead on removal	0
		2	None	Normal cocoon	+
25:1	5 CO+1 air	6	None	Dead on removal	0
20:1	4 CO+1 air	1	None	Normal cocoon	+
15:1	3 CO+1 air	5	Flat sheet	None	+
		4	Flat sheet	Normal cocoon	+
		2	Flat sheet	None	+
		1	Flat sheet	None	+
5:1	1 CO+1 air	3	Flat sheet	Normal cocoon	+
3:1	0.67 CO+1 air	5	Flat sheet	None	+
2:1	0.4 CO+1 air	3	None	Normal cocoon	+
1:1	0.2 CO+1 air	3	None	Normal cocoon	+
		3	Flat sheet	Normal cocoon	+
		3	Flat sheet at first, then continued with a normal cocoon	None	+

6. *Effects of carbon monoxide on fertile eggs and embryos*

Embryonic development of Cecropia, from oviposition to hatching, requires about ten days at 25° C. From the sixth to the tenth day, one can easily track the progress of embryonic development under the dissecting microscope and thereby estimate the stage of embryonic development.

By means of the static pressure method, freshly oviposited fertile eggs were exposed to a carbon monoxide/oxygen ratio of 20:1 for 1, 3, and 5 days, respectively. Similar experiments were performed on developing embryos which had already completed 10, 30, 70, and 90 per cent of embryological development.

In control experiments compression with four atmospheres of nitrogen was without major effects on viability, and approximately 90 per cent of eggs and embryos hatched. However, even one day of exposure to 20:1 carbon monoxide/oxygen considerably decreased the viability of the embryos. When returned to air, only 10 per cent of the eggs eventually hatched and only 50 per cent showed any detectable progress in embryonic development. Three days of exposure to the 20:1 mixture was lethal in nearly all cases; when returned to air, no eggs hatched and almost all of the embryos were already dead. It is clear that both the development and the viability of eggs and embryos are extremely sensitive to brief exposure to carbon monoxide.

7. *Effects of carbon monoxide on the adult moth*

By the use of the static pressure method, adult Cecropia moths, 12 to 36 hours after emergence, were exposed to various carbon monoxide/oxygen ratios for periods up to five days. The results summarized in Table IV demonstrate that the

TABLE IV

Effects of various carbon monoxide/oxygen ratios on the viability of adult Cecropia moths

CO/O_2	Gas content of chamber (atmospheres)	Number of animals	Duration of exposure (days)	Behavior in chamber	Behavior after removal from chamber
—	1 air	2	2	Fluttering	Flying
—	4 N_2+1 air	2	2	Fluttering	Flying
33:1	6.7 CO+1 air	2	2	Slight tremors which ceased after 2 hours	Flaccid upon removal. Recovery after 1 and 10 minutes. Feeble coordinated motion within 3 hours, but no flight. Died within 4 days
20:1	4 CO+1 air	2	5	Slight tremors which ceased after 2 hours	Flaccid and dead upon removal
		4	3	Slight tremors which ceased after 2 hours	Flaccid upon removal. Recovery after 10, 10, 20, and 60 minutes. Extremely feeble uncoordinated activity within 3 hours. Died within 3 days without regaining coordination
		2	2	Slight tremors which ceased after 2 hours	Flaccid upon removal. Recovery after 1 and 10 minutes. Considerable coordinated activity within 3 hours, but no flight. Both lived for 6 days after removal, one female laid eggs
		8	1	Slight tremors which ceased after 2 hours	Flaccid upon removal. Recovery after 30 seconds. Flying within 3 hours

moth is definitely sensitive to carbon monoxide. After three days of exposure to a carbon monoxide/oxygen ratio of 20:1, the insects showed considerable decrease in vitality when returned to air; exposure for five days was lethal. Equivalent compression with nitrogen had no effect.

8. Photoreversibility of the carbon monoxide inhibition

Six pupae showing early adult development were placed head-down in an air-filled Lucite tank such as illustrated in Figure 1, and compressed with carbon monoxide to a final carbon monoxide/oxyen ratio of 20:1. Three individuals were illuminated continuously with a 250-watt mercury vapor lamp (General Electric AH-5) *via* their terminal abdominal windows. The light was collected with a reflector and passed through a solution of sodium nitrite to cut off the ultraviolet and through 5 cms. of water to eliminate the infra-red (Bowen, 1949). Three control animals were loosely wrapped in aluminum foil to maintain them in darkness, and

placed in the same chamber. The latter was immersed in a water bath at 25° C., the distance from the light source to the animals being approximately 25 cms.

Exposure to carbon monoxide and simultaneous illumination were continued for 5 days. The chamber was then decompressed and the experimental animals compared with the controls. The genitalia of the illuminated animals had progressed an average of 3.5 days; that is, at 70 per cent of the rate in air. By contrast, the genitalia of the unilluminated individuals showed no detectable progress. This difference was particularly striking at the anterior and posterior ends of the illuminated animals in that the illuminated genitalia showed considerable progress in development whereas the unilluminated facial region showed no morphological advance. Since light-reversibility is a distinguishing property of carbon monoxide's inhibition of cytochrome oxidase, the demonstration of light-reversibility is especially critical, confirming for the insect as a whole the phenomenon as previously encountered in cultures of isolated Cecropia spermatocytes (Schneiderman, Ketchel and Williams, 1953).

9. Effects of oxygen tension on animals at the initiation of adult development

Pupae showing the first day of adult development were exposed to continuously flowing mixtures of oxygen and nitrogen for specific periods, usually 21 days. The results recorded in Table V show that development was retarded by 13 per cent in

TABLE V

Effects of oxygen tension on the adult development of Cecropia (animals on first day of development at outset)

Oxygen tension (per cent of an atmosphere)	Number of animals	Days in gas mixture	Average rate of development as per cent of rate in air	Average rate of development after return to air	Final state
100	3	21	90	2 at 100% normal rate; 1 at 60% normal rate	1 normal adult; 2 slightly abnormal
21 (air)	3	21	100	100%	Normal adults
5	3	21	87	100%	Normal adults
3	3	21	52	100%	Abnormal adults
1	3	15	0	After 10 days in air, development began again and continued at 100% normal rate	1 adult with minimal defects in antennal structure; 2 dead after 45% and 70% development
Less than 0.5	3	7	0	0	Dead when removed from gas

5 per cent of an atmosphere of oxygen, and by 10 per cent in an atmosphere of pure oxygen. Between these limits the rate of development was independent of oxygen tension. Evidently, a gradient in oxygen pressure slightly in excess of 5 per cent of an atmosphere is sufficient to meet the oxygen requirements of the developing tissues. Those individuals which underwent development in the presence of oxygen pressures less than 5 per cent showed abnormalities similar to those encountered after exposure to carbon monoxide (*cf.* section 4).

10. Effects of oxygen tension on mature larvae

Eleven mature larvae were subjected for one to four days to specific low oxygen tensions established by the flow method. The effects were judged in terms of the insect's spinning behavior and subsequent pupation.

Essentially normal cocoons were spun until the oxygen tension was reduced below 3 per cent of an atmosphere. At 2.5 per cent oxygen the animal usually spun silk in a flat sheet (*cf.* section 5). At tensions lower than 2 per cent, spinning ceased; however, animals that had been exposed to this low tension for three days spun normal cocoons when returned to air.

11. Effects of anoxia on larvae, diapausing pupae, and adults

Mature larvae and adult moths were killed by one day of exposure to tank nitrogen containing less than 0.5 per cent oxygen. When diapausing pupae were treated in like manner, the heart ceased to beat after 4 to 7 hours. Half the animals were dead after 72 hours; the survivors, when returned to air, showed resumption of heart beat and abdominal motion after one to two days.

Discussion

1. Systematic changes in sensitivity to carbon monoxide

In the preceding paper of this series, evidence derived from respiratory studies on the Cecropia silkworm demonstrated that marked changes occur in the sensitivity of respiration to carbon monoxide during embryonic and post-embryonic development. The results of the present study reaffirm these changes by demonstrating that diverse physiological activities of the insect show parallel variations in sensitivity to carbon monoxide. In the analysis of these findings it is convenient to subdivide the physiological activities of the insect into processes concerned with "maintenance" and with "growth and activity." The first of these include the minimal metabolic events which sustain the viability and *status quo* of the organism. The second category includes physiological processes responsible for morphogenesis and similar highly involved and specialized activities.

Prolonged survival in the presence of high pressures of carbon monoxide signifies that the gas fails to block the function of any tissue or organ required for the maintenance of life. Death signifies that the function of at least one such tissue or organ is blocked by carbon monoxide. In these terms it is clear that both the maintenance and the growth-and-activity processes are blocked by carbon monoxide in the egg, embryo, and larva. After pupation, however, the maintenance and survival of the diapausing pupa in the dormant state are insensitive to carbon monoxide.

The carbon monoxide-stable mechanism apparently remains intact during the early stages of adult development. But, here also, carbon monoxide continues to block development and to inhibit the contraction of all muscles except the heart. Finally, in the late stages of adult development and in the adult moth, carbon monoxide once again interferes with maintenance as well as with growth and activity.

Evidence has heretofore been presented that the target of carbon monoxide in the insect is cytochrome oxidase (Schneiderman and Williams, 1954). The light-reversibility of carbon monoxide's inhibition of growth is strong confirmation of this

view. Moreover, as was inferred in the previous study, the ability of the diapausing pupa to survive in the presence of high concentrations of carbon monoxide signifies that the loss or inactivation of the carbon monoxide-sensitive cytochrome oxidase system at the time of pupation is compensated by the development of activation of a carbon monoxide-stable respiratory system capable of underwriting the maintenance requirements and the heart-beat of the diapausing insect. This finding affords a remarkably clear illustration in biochemical terms of the dissociability of "maintenance" and "growth" (Needham, 1942, p. 505 ff.).

2. The cytochrome-cytochrome oxidase system and the energetics of development

The dependency of the growth and activity processes of Cecropia at all stages of development upon respiration mediated by cytochrome oxidase finds many parallels. From a study of the literature we have assembled in Table VI a number of processes

TABLE VI

Vital processes in which the inhibitory action of carbon monoxide has been found to be reversed by light

Material		Reference
1. *Arbacia* eggs	Cell division (mitosis)	Clowes and Krahl (1940)
2. Cecropia spermatocytes	*In vitro* spermatogenesis (meiosis and spermiogenesis)	Schneiderman, *et al.* (1951, 1953)
3. *Drosophila*	Adult development	Wolsky (1937)
4. *Avena* (oat)	Growth of isolated coleoptile sections (cell elongation)	Hackett and Schneiderman (1953)
5. *Pisum* (pea)	Growth or isolated stem sections (cell elongation)	Hackett and Schneiderman (1953)
6. *Solanum* (white potato)	Water uptake by tissue slices	Hackett *et al.* (1953)
7. *Daucus* (carrot)	Salt accumulation by tissue slices	Weeks and Robertson (1950)
8. Rat	Incorporation of radioiodine in surviving thyroid tissue	Schachner *et al.* (1943)
9. *Pteridium* (bracken sperm)	Movement of bracken spermatozoids	Rothschild (1951)
10. *Fundulus* (fish) heart	Heart-beat	Fisher and Cameron (1936, 1938)
11. Frog nerve	Action potential	Schmitt (1930)

where a light-reversible carbon monoxide inhibition has been reported. These include meiosis, mitosis, differentiation, cell elongation, water uptake, salt accumulation, flagellar movement, and nerve conduction. As Lemberg and Legge (1949) have reasoned (p. 383): "Whether the respiration of the resting cell is always catalyzed by the cytochrome system or not, it has become increasingly clear that the functional activity of the cell depends on this system." See also Drabkin (1948).

For our present purposes it is of special interest that the inhibition of cytochrome oxidase within the post-diapausing Cecropia establishes and enforces an artificial diapause during the period of exposure of carbon monoxide. It is also noteworthy that even in the diapausing pupa the inhibition of this enzyme prevents wound-healing. From these several lines of evidence we learn that carbon monoxide-sensitive metabolism plays an obligatory role in the energetics of development.

The absence of all but a trace of a complete cytochrome oxidase system in the diapausing pupa therefore assumes special significance (Williams, 1951). Since the presence and function of this system appear to be prerequisite for adult development, its virtual absence in the dormant pupa can, in itself, account for the developmental stand-still of diapause.

In diapausing embryos of the grasshopper, *Melanoplus*, and of the commercial silkworm, *Bombyx*, the absence of a cytochrome-mediated respiration has been attributed to an inactivation of the cytochrome oxidase that is already present; the oxidase is thought to be re-coupled to metabolism in synchrony with the termination of diapause (Bodine and Boell, 1938; Wolsky, 1949). But, in the case of the Cecropia silkworm, the termination of diapause and the onset of development are accompanied by an actual synthesis of a new cytochrome system—not a mere recoupling of enzymes already present (Sanborn and Williams, 1950). The results of the present investigation therefore link the respiratory and enzymatic studies and demonstrate that cytochrome oxidase is the terminal oxidase in processes energizing the insect's development.

The present study confirms the fact that qualitative as well as quantitative changes occur in the energy metabolism of the Cecropia silkworm during the course of metamorphosis. It also contributes to a coherent body of evidence that the cytochrome oxidase system plays an obligatory role in the energetics of morphogenesis. We are therefore persuaded that the recruitment and resynthesis of the cytochrome oxidase system are among the biochemical changes set in motion by the growth and differentiation hormone—changes which couple the endocrine action to the termination of the pupal diapause.

The experiments reported in Sections 5 and 10 were performed in collaboration with Dr. William Van der Kloot and those in Section 9 in collaboration with Mr. Roger Milkman. The photograph in Figure 1 was made by Dr. Roman Vishniac and is used with the permission of *Time*, Inc.

Summary

1. The effects of mixtures of carbon monoxide and oxygen on the growth and metamorphosis of the Cecropia silkworm were examined at successive stages of embryonic and post-embryonic development.

2. Embryos, mature larvae, and adults are killed by five days of exposure to carbon monoxide/oxygen ratios of 20 : 1 or 25 : 1. Diapausing pupae, by contrast, survive at least 21 days of exposure to carbon monoxide/oxygen ratios as high as 33 : 1.

3. While failing to interfere with the viability of diapausing pupae, carbon monoxide blocks or greatly retards the termination of the pupal diapause; it also inhibits the healing of experimental wounds in the pupal integument.

4. The ability to survive in the presence of high pressures of carbon monoxide persists throughout the early stages of adult development. Exposure of the developing, post-diapausing insect to suitable pressures of carbon monoxide establishes and enforces an artificial diapause which is reversed upon return to air.

5. The inhibition of adult development by carbon monoxide is light-reversible; the degree of inhibition is a function of the carbon monoxide/oxygen ratio. These

findings indicate that the effects of carbon monoxide are due to the poisoning of cytochrome oxidase.

6. Resistance to carbon monoxide, as in the diapausing pupa, signals the presence and utilization of an oxidase other than cytochrome oxidase.

7. On the basis of these several lines of evidence, it is concluded that growth and metamorphosis, at all stages in the life history, are dependent on metabolism catalyzed by cytochrome oxidase. The function of cytochrome oxidase is likewise prerequisite for the maintenance of life of the embryo, larva, and adult.

8. Only the diapausing pupa survives without regard to the presence or function of cytochrome oxidase, the maintenance metabolism of the pupae being served by an unidentified oxidase which is insensitive to carbon monoxide.

9. With the termination of pupal diapause the growth and differentiation of the adult moth again requires the function of the cytochrome oxidase system. This fact is considered in relation to the endocrine control of the pupal diapause.

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